Supplemental Material

Figure S1

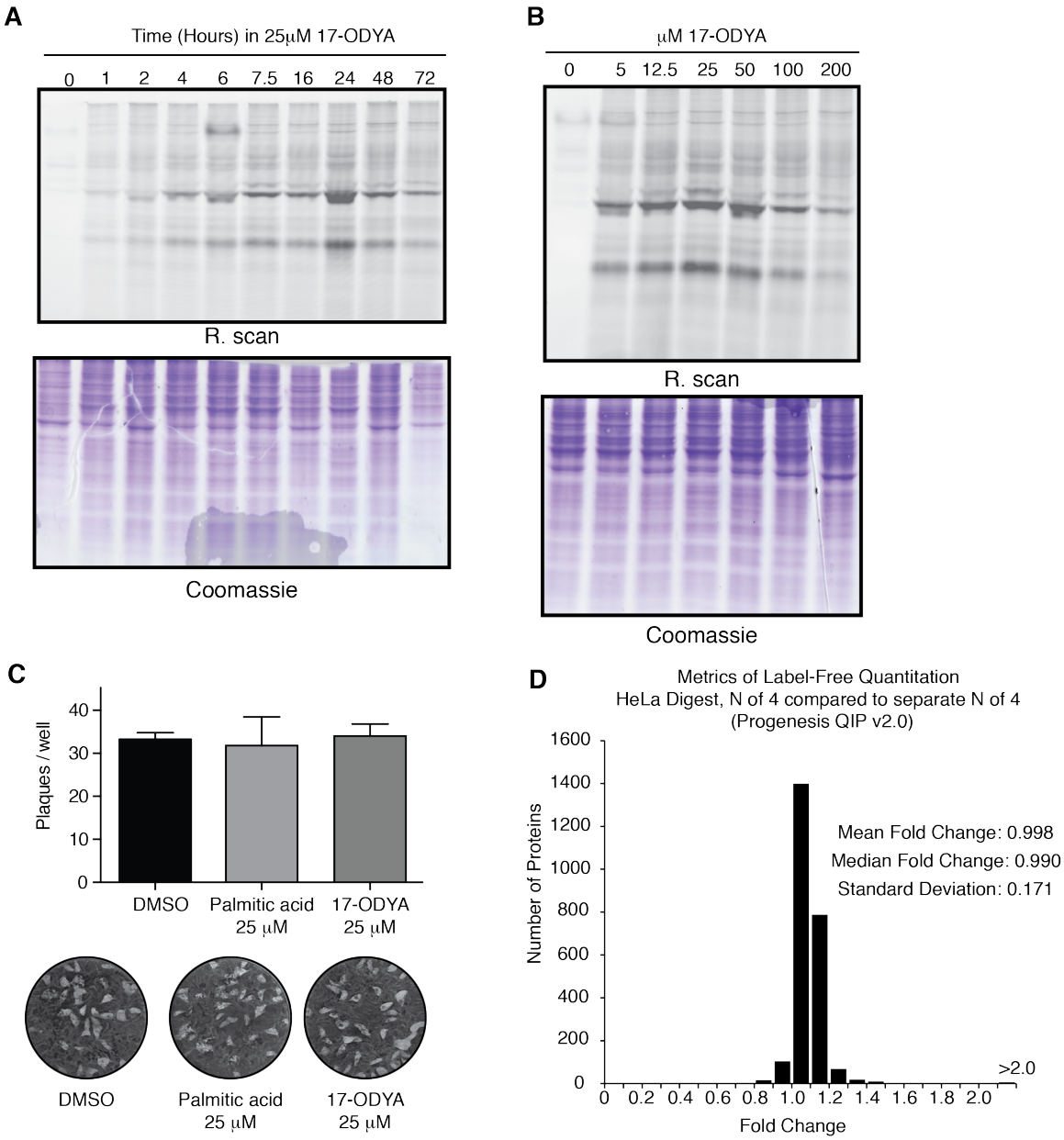


Figure S2

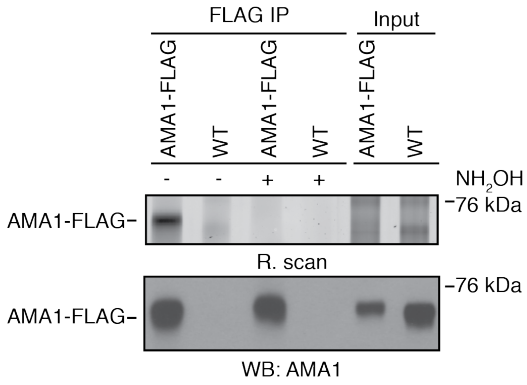


Figure S3

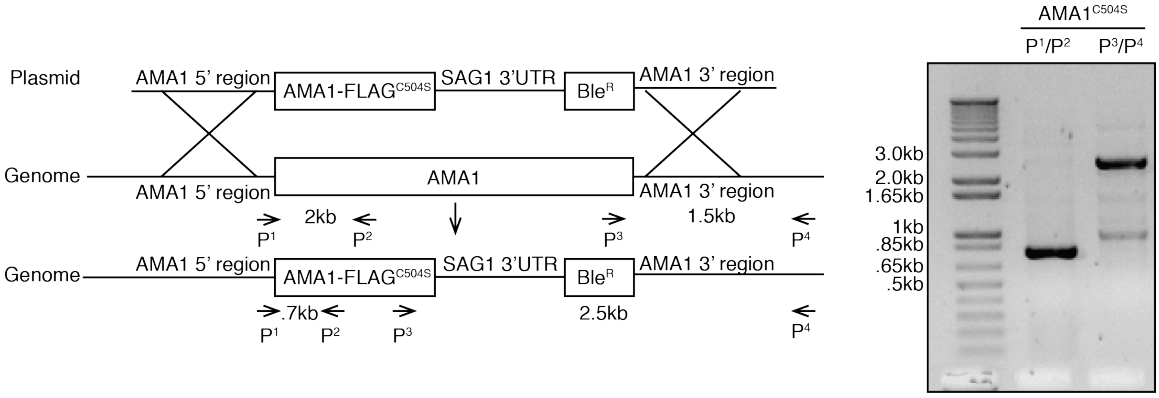
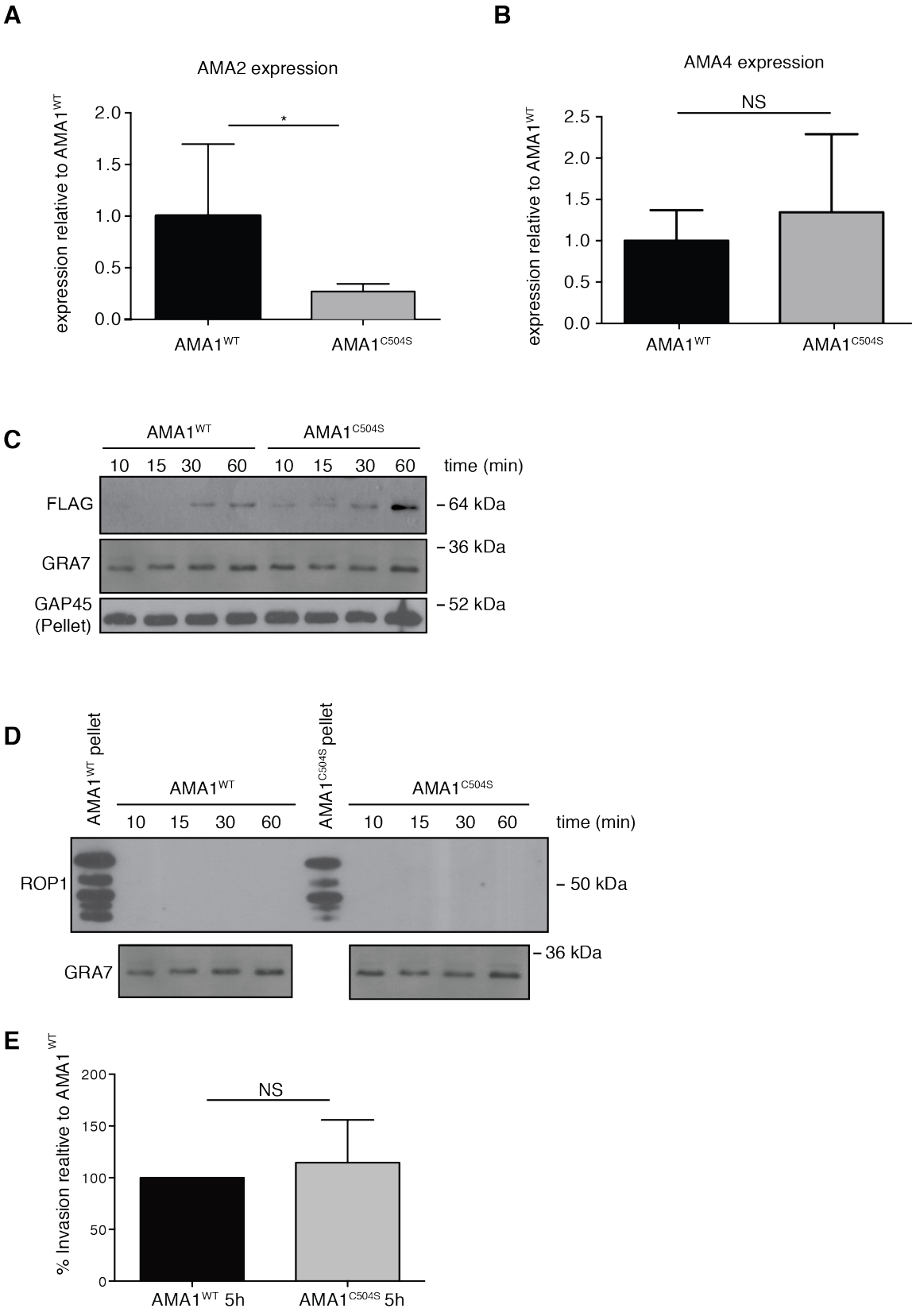


Figure S4


A
AMA2 expression
expression relative to AMA1WT
2.0
1.5
1.0
0.5
0.0
*
AMA1WT
AMA1C504S
B
AMA4 expression
NS
expression relative to AMA1WT
2.5
2.0
1.5
1.0
0.5
0.0
AMA1WT
AMA1C504S
C
AMA1WT
AMA1C504S
10 15 30 60 10 15 30 60
time (min)
FLAG
64 kDa
GRA7
36 kDa
GAP45
(Pellet)
52 kDa
D
AMA1WT pellet
AMA1C504S pellet
AMA1WT
AMA1C504S
10 15 30 60 10 15 30 60
time (min)
ROP1
50 kDa
GRA7
36 kDa
E
% Invasion realtive to AMA1WT
200
150
100
50
0
NS
AMA1WT 5h
AMA1C504S 5h

Supplemental Figure Legends

Figure S1, related to Figure 1. Optimization of metabolic labeling with 17-ODYA. **A.** Time course of labeling with 25mM 17-ODYA over 72 hours. Top panel shows incorporation of 17-ODYA by rhodamine fluorescence (R. scan). Time indicated in hours. Bottom panel is a coomassie staining of the same gel to show loading. **B**. Titration of 17-ODYA to find optimal concentration for labeling over a 16 hour period. Top panel shows incorporation of 17-ODYA by rhodamine fluorescence (R. scan). Concentrations tested indicated. Bottom panel is a coomassie staining of the same gel to show loading. **C**. 17-ODYA at 25 μM has no effect on *T. gondii* growth. Top, histogram presenting the quantification of plaque assays for parasites grown for 7 days in; DMSO, 25 μM palmitic acid, and 25 μM 17-ODYA. Data presented is the average of 4 technical replicates, error bars indicate +/- standard deviation of the mean. Bottom, representative images of plaque assays. **D.** Accuracy and precision of data-independent acquisition label-free quantitation. A HeLa tryptic digest (Promega) was analyzed in 8 separate injections using the same acquisition parameters and label-free quantitative analysis. Cross-comparison of 2 groups of 4 replicates by label-free quantitation in Progenesis QiP provides a measure of technical variation, reporting accuracy within a fraction of 1% and precision near 17%.

Figure S2, related to Figure. 3. AMA1 is palmitoylated when parasites are intracellular. AMA1-FLAG IP from intracellular parasites, showing that AMA1 is palmitoylated when parasites are intracellular. Top panel: rhodamine fluorescent scan (R. scan) of SDS-PAGE showing palmitoylation of AMA1. Bottom panel: corresponding western blot for AMA1 IP, probed with anti AMA1 antibody (WB: AMA1).

Figure S3, related to Figure 4. Diagram of how AMA1^{C504S} was created by allelic replacement. Left, diagram of plasmid and genomic locus shown. The position of primers used to determine correct integration are indicated as well as expected product size. Right, diagnostic PCR showing correct integration of the replacement cassette.

Figure S4, related Figure 5. A. AMA2 is down-regulated in AMA1^{C504S} strain. Histogram showing average AMA2 transcript levels in AMA1^{C504S} strain relative to AMA1^{WT} determined by qPCR. Histogram presents data for the mean of 2 biological replicates, each performed in technical triplicate. Error bar represents standard deviation of the mean. Statistical significance determined by Student's t-test, * p = 0.029. **B**. AMA4 is not up-regulated in AMA1^{C504S} strain. Histogram showing average AMA4 transcript levels in AMA1^{C504S} strain relative to AMA1^{WT} determined by qPCR. Histogram presents data for the mean of 2 biological replicates, each performed in technical triplicate. Error bar represents standard deviation of the mean. Statistical significance determined by Student's t-test, NS, not significant. **C.** Representative time course of constitutive microneme secretion comparing AMA1^{WT} with AMA1^{C504S}. The anti-FLAG blot (top) indicates differences in constitutive secretion of AMA1, with the anti-GRA7 blot (middle) presented as a soluble loading control for constitutive dense granule secretion. Intact parasite pellet samples generated during the assay are used to control for parasite number in each sample, with the pellets from each timepoint probed for GAP45 as an insoluble parasite marker (bottom, Pellet). **D**. Rhoptry proteins are not secreted in either the AMA1^{WT} or the AMA1^{C504S} strain. Anti-ROP1 blot shows the basal

rate of ROP1 secretion. GRA7 recovered in the assay supernatant was used as a control. **E.** $AMA1^{C504S}$ parasites left extracellular for 5 hours invade host cells normally. Histogram presenting the quantification of 5 independent invasion assays using parasites that were maintained extra cellularly at 23°C for 5 hours. Each assay was done in technical duplicate. Percent invasion shown for $AMA1^{C504S}$ relative to $AMA1^{WT}$ invasion, NS indicates not significant by Student's t-test.

Supplemental Table Legends

Table S1, related to Figure 1 and Figure 2. All proteins identified and filtered to obtain the total palmitome. Sheet 1: (Dataset). Data set shows all 834 non-ambiguous proteins identified. **Sheet 2:** (ODYA enriched) Shows 501 proteins that were enriched in 17-ODYA over palmitic acid by at least 1.5 fold and were statistically significant (P value < .05) and satisfied 5% FDR. Proteins that insensitive to hydroxylamine are highlighted in green. **Sheet 3:** (Highly hydroxylamine sensitive) 210 proteins that were enriched in 17-ODYA over hydroxylamine by at least 1.5 fold and are statistically significant and satisfied 5% FDR. **Sheet 4:** (Hydroxylamine sensitive) 73 proteins that were enriched in 17-ODYA over hydroxylamine between 1.5 and 1 fold and were statistically significant, and satisfied 5% FDR **Sheet 5:** (Ambiguous proteins identified) Proteins that could not be unambiguously identified based on protein sequence.

Table S2, related to Figure 2. Overlap between the *T. gondii* palmitome and the *P. falciparum* palmitomes. **Sheet 1:** Proteins that overlap between the *T. gondii* and Total *P. falciparum* palmitome are shaded blue. *P. falciparum* genes and their *T. gondii*

orthologue pairs indicated. **Sheet 2:** Proteins that overlap between the *T. gondii* and 17-ODYA *P. falciparum* palmitome are shaded blue. *P. falciparum* genes and their *T. gondii* orthologue pairs indicated.

Supplemental Experimental Procedures

Parasite growth conditions. Parasites were grown on human foreskin fibroblast (HFFs), in DMEM with 10% FBS, 2mM L-glutamine, 100μg/ml Penicillin, and 100μg/ml Streptomycin. Parasites were cultured at 37°C in 5% CO_2. Parasites were collected either by manual lysis of HFF or by collection of parasites from the supernatant of lysed HFF.

17-ODYA synthesis. 17-ODYA was synthesized from oleic acid using previously described methods (Augustin and Schäfer, 1991; Menger et al., 1993).

Plaque assays for 17-ODYA toxicity.

Fifty RH parasites were added to each well of a 12-well plate containing confluent host cell HFF monolayers with either DMSO, 25 μM palmitic acid, or 25 μM 17-ODYA added to media. The plate was incubated for 7 days in the 37°C incubator with 5% CO_2 and humidity, then stained with 2% crystal violet and 20% methanol in PBS for 5 minutes at 23°C. The stained wells were washed with water and the number of plaques per well counted.

LC-MS analysis. Samples were prepared as previously reported (Martin et al., 2012). Tryptic digests from the *Toxoplasma gondii* samples were injected onto a 1D Waters NanoAcquity UPLC system equipped with a 5 μM Symmetry C18 (180 μS x 20 mm,

Waters) trap column and a 1.8 μ x High Strength Silica (HSS-T3) analytical column (75 μ H x 150 mm, Waters). Tryptic peptides were loaded onto the trap column over 3 minutes, followed by analytical separation over a 110 minute gradient (3% acetonitrile to 40% acetonitrile over 92 minutes). Peptides were analyzed using a Waters Synapt G2S HDMS time-of-flight mass spectrometer with (UDMS^{E} mode) traveling-wave ion mobility separation and data independent fragmentation algorithms. Briefly, the instrument was tuned using the following parameters. The triple-quadruple mass analyzer was manually set for *m/z* 500, 600 and 700. The sampling cone was adjusted to 32 eV and the nano flow gas was set to flow at 0.2 bar. The purge gas was set to flow at 50 L / h and the source temperature was set at 70 °C. For all measurements, the mass spectrometer was operated in V-mode (resolution mode) with a resolving power of at least 20,000 FWHM (full width at half maximum) in positive-mode ESI. The time-of-flight analyzer of the mass spectrometer was calibrated with a 100 fmol / µL solution of [Glu1]-Fibrinopeptide B from *m/z* 50 to 1250 to within 0.7 ppm. Post-acquisition lock mass correction was performed using the doubly charged monoisotopic ion of [Glu1]-Fibrinopeptide B (*m/z* = 785.8426), collected every 30 seconds throughout the run. For ion mobility separation (IMS), the wave height was set as 40 V and IMS wave velocity as 600 m/s. The spectral acquisition time in each mode was 0.5 s. In low-energy MS mode, data were collected without applying collision energy in the trap or the transfer stage. Briefly, the following CE settings were defined and used throughout the studies in the elevated energy scan: (i) ion-mobility bins 0 – 20: CE of 117 eV, (ii) ion-mobility bins 20 – 110: CE of 17 eV to 56 eV, (iii) ion-mobility bins 110 – 200: 56 to 72 eV. All data was collected as three technical replicates for each of 4 biological replicates.

Label-free quantitation and filtering. Raw files acquired using UDMS^{E} methods were loaded in ProgenesisQIP v 2.0 (Waters) for both identification (using PLGS) and label-free quantitation. ProgenesisQIP v2.0 cross-extracts peaks across replicates, thus increasing the depth of coverage. PerogenesisQIP v2.0 aligns the various replicates using a vector-based algorithm. Peak picking was performed using Apex3D, a data-independent analysis algorithm. A "raw abundance" value is calculated for each *m/z* feature across replicates based on the area under the curve represented by the feature. Quantitation prior to identification is performed by comparison of this abundance value with the average raw abundance value in the other condition to provide a "fold-change" value. Post quantitation, the quantified MS1 data was searched for identifications against the toxoplasma database (downloaded from uniprot on 9/30/2014) using the following parameters: (i) minimum 2 fragment ions / peptide (ii) minimum 5 fragment ions / protein (iii) minimum 2 peptides / protein (iv) maximum allowed MS1 mass error = 10 ppm, and (v) maximum allowed MS2 mass error = 20 ppm. Post-identification, data were normalized to 43 quantified peptides of pyruvate carboxylase. The enrichment protocol in the labeling workflow is based on biotin-streptavidin affinity, and natively biotinylated proteins are common impurities between all experiment conditions and groups. To establish an internal sample-derived control, we chose to use the known biotinylated pyruvate carboxylase that was present in all samples of both the 17ODYA and the palmitic acid control groups. Uniprot gene IDs were converted to the GT1 *T. gondii* gene IDs using the ToxoDB genome browser version 13.0.

Comparison of *P. falciparum* and *T. gondii* palmitomes. *P. falciparum gene* IDs were obtained from the Supplemental Tables 2 and 4 in Jones et al., 2012. Gene IDs were converted to *T. gondii* orthologues using the transform by orthology function in EupathDB release 25 and 24 respectively.

Protein alignment. Protein sequences were obtained from EuPATH DB (TGGT1_255260 *T. gondii*, PF3D7_1133400 *P. falciparum*, PVX_092275 *P. vivax,* PBANKA_091500 *P. berghei,* PCHAS_093100 *P. chabaudi).* Proteins were aligned using the program T-Coffee (http://tcoffee.crg.cat/apps/tcoffee/do:regular) and reformatted for publication using boxshade: (http://www.ch.embnet.org/software/BOX_form.html).

Generation of transgenic parasite lines

Human foreskin fibroblasts (HFF ; ATCC CRL-1643) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10mM HEPES, 10units/ml Penicillin, 10 units/ml Streptomycin sulphate and 10% heat-inactivated FBS at pH 7.2 in a 37°C incubator with 5% CO_2 and humidity. Prior to infecting the confluent monolayers with parasites, the medium was replaced with medium containing 1% FBS.

ku80::hxgprt RH strain parasites (Huynh and Carruthers, 2009) containing FLAG-tagged wild-type TgAMA1 or the C504S mutant at the endogenous *TgAMA1* locus were generated using the allelic replacement vector pA/TgAMA1^{WT} Flag.BLE described in (Parussini et al., 2012). Around the horn mutagenesis was used to introduce the point mutation using the primers ARH C504S FP- 5'P CCTACTTC GCGAAGAGGT

TGGACAGAAAC 3' and ARH C504S RP-5'P AGCCTCCTCCTAGAAGAGCCAACAGCAG-3'**.** The vector was double digested with XhoI and SacI restriction endonuclease for transfection. Transfected parasites were selected twice with 50µg/ml Phleomycin (Ble) and maintained in 5µg/ml Ble until cloned by limiting dilution.

To measure protein expression levels, cloned parasites were boiled in Laemmli sample buffer, run on a 12% SDS-PAGE gels and transferred onto PVDF membranes. The blots were blocked overnight with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE), incubated with mouse anti-Flag and rabbit anti-TgMLC1 antibodies, followed by IRDye 800 CW-conjugated anti-mouse and IRDye 680 CS-conjugated anti-rabbit (LI-COR), washed in PBS, and scanned using an Odyssey Imaging System CLx (LI-COR).

One clone of each parasite line (expressing equivalent amounts of wild-type and mutant FLAG-tagged protein) was chosen for subsequent analysis. The *TgAMA1* open reading frame was sequenced from genomic DNA to confirm the presence or absence of the C504S mutation, and the clones were checked for integration of the plasmid at the right locus by diagnostic PCR using primers as described (Parussini et al., 2012). Localization of the transgene in intracellular parasites was determined by indirect immunofluorescence with anti-FLAG and anti-GAP45 antibodies as described (Parussini et al., 2012). Localization of the transgene in extracellular parasites was determined by indirect immunofluorescence. Parasites were syringe lysed out of host cells, allowed to sit for 1 hour extracellular at 37°C, and then were placed on ice for 20 minutes, then were spun onto HFF coated coverslips at 4°C, and fixed on ice with cold 4% paraformaldehyde and stained with anti-FLAG and anti GAP45 as described (Parussini et al., 2012).

Microneme Secretion Assays. Microneme secretions assays performed as previously described (Child et al., 2013). ROP1 western blot was performed using the Tg49 anti ROP1 antibody from John Boothroyd at 1:1000 in PBST. GAP45 westerns were done as described in (Child et al., 2013)

AMA2 and AMA4 quantitative PCR.

Parasite RNA was isolated using trizol reagent. Reverse transcription was performed using either Qiagen's Quantitect reverse transcription kit or Invitrogen's superscript III. Quantitative PCR was performed using Clontech Sybr advantage QPCR mix on a Stratagene MX3000 using Actin as a standard and was performed for AMA2 and AMA4 using the primers and conditions described previously (Lamarque et al., 2014).

References

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